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Influence of Long Terminal Repeat and Env on the Virulence Phenotype of Equine Infectious Anemia Virus

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The molecular clones pSPeiav19 and p19/wenv17 of equine infectious anemia virus (EIAV) differ in *env* and long terminal repeats (LTRs) and produce viruses ($EIAV_{19}$ and $EIAV_{17}$, respectively) of dramatically different virulence phenotypes. These constructs were used to generate a series of chimeric clones to test the individual contributions of LTR, surface (SU), and transmembrane (TM)/Rev regions to the disease potential of the highly virulent $EIAV_{17}$. The LTRs of $EIAV_{19}$ and $EIAV_{17}$ differ by 16 nucleotides in the transcriptional enhancer region. The two viruses differ by 30 amino acids in SU, by 17 amino acids in TM, and by 8 amino acids in Rev. Results from in vivo infections with chimeric clones indicate that both LTR and *env* of $EIAV_{17}$ are required for the development of severe acute disease. In the context of the $EIAV_{17}$ LTR, SU appears to have a greater impact on virulence than does TM. $EIAV_{17SU}$, containing only the TM/Rev region from the avirulent parent, induced acute disease in two animals, while a similar infectious dose of $EIAV_{17TM}$ (which derives SU from the avirulent parent) did not. Neither $EIAV_{17SU}$ nor $EIAV_{17TM}$ produced lethal disease when administered at infectious doses that were 6- to 30-fold higher than a lethal dose of the parental $EIAV_{17}$. All chimeric clones replicated in primary equine monocyte-derived macrophages, and there was no apparent correlation between macrophage tropism and virulence phenotype.

Equine infectious anemia virus (EIAV) is a macrophage-tropic lentivirus of horses (42). The virus differs from the feline, simian, and human immunodeficiency viruses (FIV, SIV, and HIV, respectively) in that lymphocytes are not targets of virus replication and that infected animals do not develop severe immunodeficiency (32). Following exposure to the virus, an acute disease episode of relatively short duration generally occurs within 1 week to 1 month. Acute disease is characterized by fever, anemia, thrombocytopenia, central nervous system depression, and anorexia (reviewed in references 19 and 41). The acute disease episode is generally mild, but horses will occasionally develop a particularly severe, fatal form of acute disease that is characterized by a high-titer viremia and very high body temperature. Animals recovering from the acute disease episode may enter the chronic or persistent phase of EIA, which is characterized by periodic episodes of clinical illness. Most infected horses gain immunologic control of virus replication, becoming inapparent carriers.

EIAV antigenic and genomic variations have been previously examined at length (1, 3, 6, 17, 18, 21, 23, 24, 33, 35, 39, 40, 45, 46). Studies of *env* gene variation demonstrate that infected animals maintain the virus as a quasispecies, as has been previously documented for other lentiviruses (17, 18, 23, 24, 26, 34, 35, 45, 46). Accumulation of mutations in specific variable domains of the surface (SU) unit protein allows the virus to evade or escape neutralizing antibodies (14, 15). Genetic variation in other regions of the EIAV genome has also been described previously. We and others have demonstrated the influence of long terminal repeat (LTR) sequences on the growth characteristics of EIAV (25, 31, 36). Genetic analysis of *rev* during long-term infection suggests that *rev* variation may influence virus expression, affecting long-term viral persistence (3, 4).

Previous studies of EIAV genetic variation have focused largely on understanding the virus-host interactions that result in chronic disease and viral persistence rather than on defining the determinants of virulence. For the present study, we used infectious molecular clones of EIAV to examine the genetic basis of two very different virulence phenotypes that have been described previously (37, 38). $EIAV_{19}$ is a virus stock derived from the infectious molecular clone pSPeiav19. $EIAV_{19}$ replicates in primary fetal equine kidney, equine dermis, and equine monocyte-derived macrophages (eMDM) as well as in the feline embryonic adenocarcinoma and Cf2Th cell lines (38). We have observed that $EIAV_{19}$ causes no clinical disease, even when administered to ponies at high doses (38).

To generate a genetically defined virulent virus, pSPeiav19 was used as the backbone to produce a set of clones in which *env* and LTRs were replaced by sequences obtained directly from the highly virulent Wyoming field strain of EIAV (37). One construct, p19/wenv17, has been described previously, and virus derived from this clone (designated $EIAV_{17}$) causes severe acute disease in our Shetland pony model (37). $EIAV_{17}$

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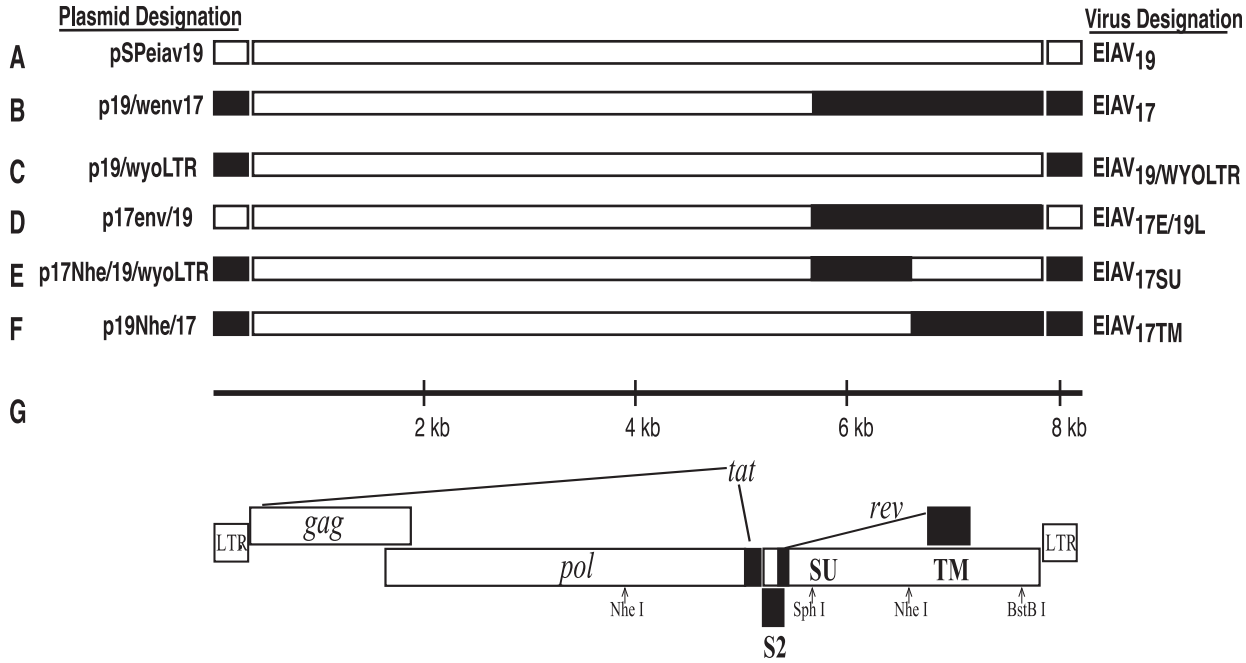

FIG. 1. Schematic representation of clones and the EIAV genome. (A through G) Open boxes represent the sequence of pSPeiav19 (GenBank accession number U01866 [38]). Solid black boxes represent sequences derived from the Wyoming field strain that were used to generate the p19/wenv17 clone (GenBank accession number AF028232 [37]). Plasmid designations are shown to the left of each construct; virus designations are shown to the right. Panel G shows the relative positions of EIAV open reading frames. The restriction enzyme sites used to generate new clones are indicated.

replicates only in eMDM, a characteristic shared by field strains of EIAV (5). The acute virulence phenotype of $EIAV_{17}$ is useful for studies in which the availability of experimental animals is limited.

Sequence differences between clones pSPeiav19 and p19/wenv17 are limited to an approximately 2-kb fragment of *env* and the viral LTRs. Virus stocks derived from both pSPeiav19 and p19/wenv17 replicate to a high titer, with cytopathic effects, in eMDM (37), so it is unlikely that the dramatic difference in their virulence phenotypes is due solely to macrophage tropism. These clones provide an ideal starting point to locate those genes or regions that play major roles in virulence. In this report, we describe the construction and testing of several new infectious molecular clones of EIAV that we have used to determine the contributions of *env* and LTRs to the virulence phenotype of $EIAV_{17}$ and demonstrate that both SU and LTRs contain virulence determinants.

MATERIALS AND METHODS

Clone construction. Infectious molecular clones described in this report are depicted in Fig. 1. pSPeiav19 (Fig. 1A), p19/wenv17 (Fig. 1B), and p19/wyoLTR (Fig. 1C) have been described previously (36–38). The new construct p17env/19 (Fig. 1D) contains an *env* gene fragment from p19/wenv17, but its LTRs are derived from pSPeiav19. p17env/19 was constructed by using a combination of PCR and conventional cloning. PCR was used to generate a 338-bp fragment containing nucleotides (nt) 7910 to 8231 from pSPeiav19 using primers WS1 (5′-TGTGGGGTTTTTATGAGGGG-3′) and 8211R1 (5′-CGCGGTCGACGA ATTCTGTAGGATCTCGAACAGAC-3′) (Fig. 2). Primers 7582 (5′-TAAGG ACCCTTGGTG-3′) and 91 (5′-GCCTAGCAACATGAGCT-3′) were used to generate a 428-bp fragment (nt 7587 to 8014) from p19/wenv17. The two PCR products overlap by 107 identical nt between the oligonucleotides WS1 and 91. These two products, and primers 8211R1 and 7611 (5′-CCCCCCCCGTCGAC

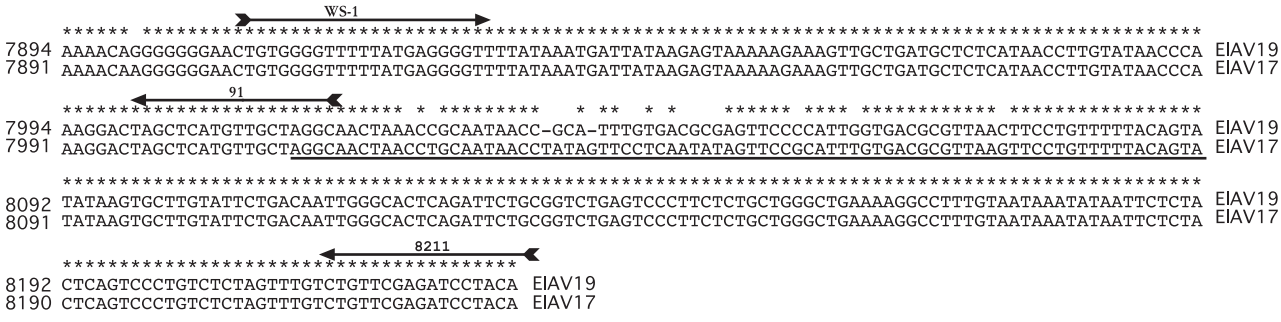

FIG. 2. $EIAV_{19}$ and $EIAV_{17}$ LTR sequences. The underlined region is the transcriptional enhancer. The sequence differences in this region result in the presence of a unique set of transcription factor binding sites for each virus; similar EIAV LTRs have been previously described and mapped by others (28, 29). The arrows represent oligonucleotide primers used in PCR to facilitate the construction of p19/wyoLTR. The numbering scheme is based on the position of the 3′ LTR from clones pSPeiav19 and p19wenv/17.

GAGATTCGAAGCGAAGG-3′), were used to amplify a 636-bp fragment that places the pSPeiav19 LTR onto the 3′ end of the *env* region of p19/wenv17. The 636-bp fragment was digested with *Bst*BI and *Eco*RI and was used to replace the corresponding region from a 2.6-kbp (*Sph*I to *Eco*RI) fragment of p19/wenv17 (37). The new 2.6-kbp *Sph*I-to-*Eco*RI fragment was then moved into pSPeiav19 in order to generate p17env/19.

To construct p17Nhe1/19/wyoLTR (Fig. 1E), we started with p19/wyoLTR (36), which had been digested with *Nhe*I to remove a 2.79-kbp *pol-env* fragment (Fig. 1G). The corresponding *Nhe*I fragment was isolated from p19/wenv17 and ligated to the prepared vector. The two *Nhe*I fragments share sequences from the 5′ *Nhe*I site through the *Sph*I site but differ from the *Sph*I site to the 3′ *Nhe*I site; thus, the *Nhe*I fragment swap effectively places the *Sph*I-to-3′-*Nhe*I fragment from p19/wenv17 onto the p19/wyoLTR background. Clone p19Nhe/17 (Fig. 1F) derives sequences from the 3′ *Nhe*I site through the 3′ LTR from p19/wenv17. This plasmid was prepared by starting with clone p19/wenv17, removing its 2.79-kb *Nhe*I fragment, and replacing it with the corresponding *Nhe*I fragment from pSPeiav19. All clones were analyzed by restriction enzyme digestion and partial DNA sequence analysis in order to check that the desired genetic swaps and replacements were present. All clones were constructed and maintained in a derivative of the low-copy-number vector pLG338 (10).

Virus stocks. To generate infectious virus, plasmid DNA was used to transfect D17 cells (ATCC CCL-183) by using a calcium phosphate transfection kit (Invitrogen) as described previously (37), and culture supernatants were collected at 24, 48, and 72 h. Culture supernatants were tested for the presence of reverse transcriptase (RT), as described previously (13), using [^{3}H]TTP in place of [^{32}βP]TTP. One-milliliter aliquots of D17 cell supernatants were passed onto eMDM to generate virus stocks. The eMDM were recovered and maintained as described previously (44).

The 50% infective dose (ID_{50}) for eMDM was also determined for each virus stock. Cells were plated in poly-D-lysine-coated 12-well dishes (BD Biosciences), infected 4 days postplating, and maintained for 11 days postinfection (p.i.). Virus was concentrated from culture supernatants by centrifugation; the presence of virus was determined by RT assay. RT values of greater than three times that of uninfected control cultures were considered positive for virus growth.

To serve as another indicator of viral infectivity, a real-time Taqman PCR assay was used to determine the provirus copy numbers in eMDM at 24 h p.i. Cells were lysed, and total DNA was purified by using a QIAmp tissue kit (Qiagen). A Taqman real-time PCR assay was performed on a Bio-Rad Icycler machine as follows. Each 50-μl reaction contained 1× reaction buffer (Applied Biosystems) without $MgCl_2$, 5.5 mM $MgCl_2$, 0.3 mM deoxynucleotide triphosphates, 0.3 μM upstream primer (5′-TTCCCATGACAGCAAGGTTT-3′), 0.3 μM downstream primer (5′-TCCATTGTCTATATGTCTGCCTAAA-3′), 0.07 μM Taqman probe (5′-FAM-CAAAAGCAGGCTCCATCTGTCTTTCTCTAG GACT-TAMRA-3′), 5 to 10 μg of DNA template or water control, and 1.25 U of AmpliTaq Gold (Applied Biosystems). Cycling parameters were 95°C for 30 s and 58°C for 1 min (40 cycles). DNA standards consisted of pSPeiav19 plasmid DNA at 10^2 to 10^6 molecules per reaction in duplicate. Cell DNA samples were analyzed in duplicate, and real-time data were analyzed by using Bio-Rad Icycler software.

Animal studies. Animal studies complied with all relevant federal guidelines and institutional policies. Virus stocks were used to infect Shetland ponies as previously described (37). Animals inoculated with the following virus stocks received a virus dose of 2×10^5 counts per minute (cpm) of RT activity: $EIAV_{17E/19L}$, $EIAV_{19/WYOLTR}$, and $EIAV_{17TM}$. $EIAV_{17SU}$ was inoculated at a dose of 10^5 cpm of RT activity.

Rectal temperatures of infected animals were monitored twice daily. Blood samples were obtained pre- and postinfection for determination of complete blood counts. Disease episodes were defined as periods of increased body temperature. Blood and serum samples were collected to quantify virus, to monitor the development of antiviral antibody, and for recovery of viral genomes. Enzyme-linked immunosorbent assay (ELISA) to detect a transmembrane peptide was performed by using a commercial assay (obtained from Centaur, Inc.). Agar gel immunodiffusion assays were performed as described previously (9). Viral genome fragments were amplified from blood samples collected within 2 weeks p.i., cloned, and sequenced to confirm the identity of the infecting virus stocks.

RESULTS

In previous studies, we demonstrated a difference in the ability of $EIAV_{19}$ and $EIAV_{17}$—derived from two infectious molecular clones, pSPeiav19 and p19/wenv17, respectively—to produce disease in Shetland ponies. $EIAV_{19}$ fails to produce clinical symptoms, even when administered to ponies at doses of up to 10^6 cpm of RT activity (38). In contrast, $EIAV_{17}$, which contains *env* and LTR sequences derived from the Wyoming field strain of EIAV, induces severe disease by two weeks p.i. when administered at 10- to 20-fold-lower doses (37). As illustrated in Fig. 1, these two clones share approximately 5.4 kb of sequence, differing only in their LTRs (Fig. 2) and *env*/*rev* regions (Fig. 3). Regions in common include *gag*, *pol*, *tat*, S2, and the amino terminus of *env*. The shared *env* region has a length of 438 bp, encompassing the first 146 amino acids of the *env* open reading frame and the first coding exon of *rev*. To further investigate the genetic region(s) responsible for the different virulence phenotypes, we constructed additional clones combining defined regions of the pSPeiav19 and p19/wenv17 genomes.

Contribution of *env* and LTR sequences to virulence phenotype. We first assayed the individual contributions of the Wyoming-derived LTR and *env* sequences to the virulence phenotype of $EIAV_{17}$. We constructed p17env/19 (Fig. 1D), which contains two complete LTRs from the avirulent clone pSPeiav19 in combination with the *env* region from the virulent p19/wenv17. Figure 2 shows the primers used in a PCR strategy to facilitate the LTR replacement. The $EIAV_{19}$ LTR was swapped for the $EIAV_{17}$ LTR in an identical region of 107 bp that begins just 3′ to the polypurine tract. The two LTRs differ at 16 positions that fall within the enhancer region in U3 (Fig. 2) and confer changes in previously mapped transcription factor binding sites (28, 29). As shown in Table 1, $EIAV_{17E/19L}$, derived from clone p17env/19, replicates in eMDM to produce a virus stock with RT activity equivalent to that of the parental $EIAV_{17}$ virus. When tested in vivo, $EIAV_{17E/19L}$ failed to produce a febrile episode during the first 45 days p.i. (Fig. 4B); however, the infected animal seroconverted by day 18 p.i. While only one animal was inoculated with $EIAV_{17E/19L}$, the virus dose (as determined by RT activity) was twofold higher than the dose of $EIAV_{17}$ that produced severe disease episodes (requiring euthanasia) in two animals (Fig. 4A). We recently determined ID_{50} titers in eMDM for each of the virus stocks tested in animals (Table 1). By this criterion, $EIAV_{17E/19L}$ was administered at an infectious dose that was 2.5-fold higher than the lethal dose of $EIAV_{17}$, suggesting that the Wyoming-derived LTR makes an important contribution to the virulence phenotype of $EIAV_{17}$.

Clone p19/wyoLTR (Fig. 1C) derives its LTRs from the virulent parent p19/wenv17 and derives its *env*/*rev* sequences from the avirulent parent pSPeiav19, as described previously (36). As shown in Table 1, $EIAV_{19/WYOLTR}$, derived from clone p19/wyoLTR, replicates in eMDM to produce virus stocks with RT activity equivalent to that of $EIAV_{17}$. $EIAV_{19/WYOLTR}$ was used to infect two animals, whose clinical profiles are shown in Fig. 4C and D. The animals seroconverted at 19 and 21 days p.i., but neither experienced a febrile episode for the first 100 days p.i. A third animal infected with $EIAV_{19/WYOLTR}$ also failed to exhibit clinical symptoms and did not seroconvert until approximately 50 days p.i. (data not shown). $EIAV_{19/WYOLTR}$ was administered to ponies at twice the lethal dose (as measured by RT activity) of $EIAV_{17}$; however, the ID_{50} per cpm of this virus stock is approximately 10-fold less than that of the $EIAV_{17}$ stock. By this criterion, the infecting dose in each of three animals was approximately 2.5-fold lower

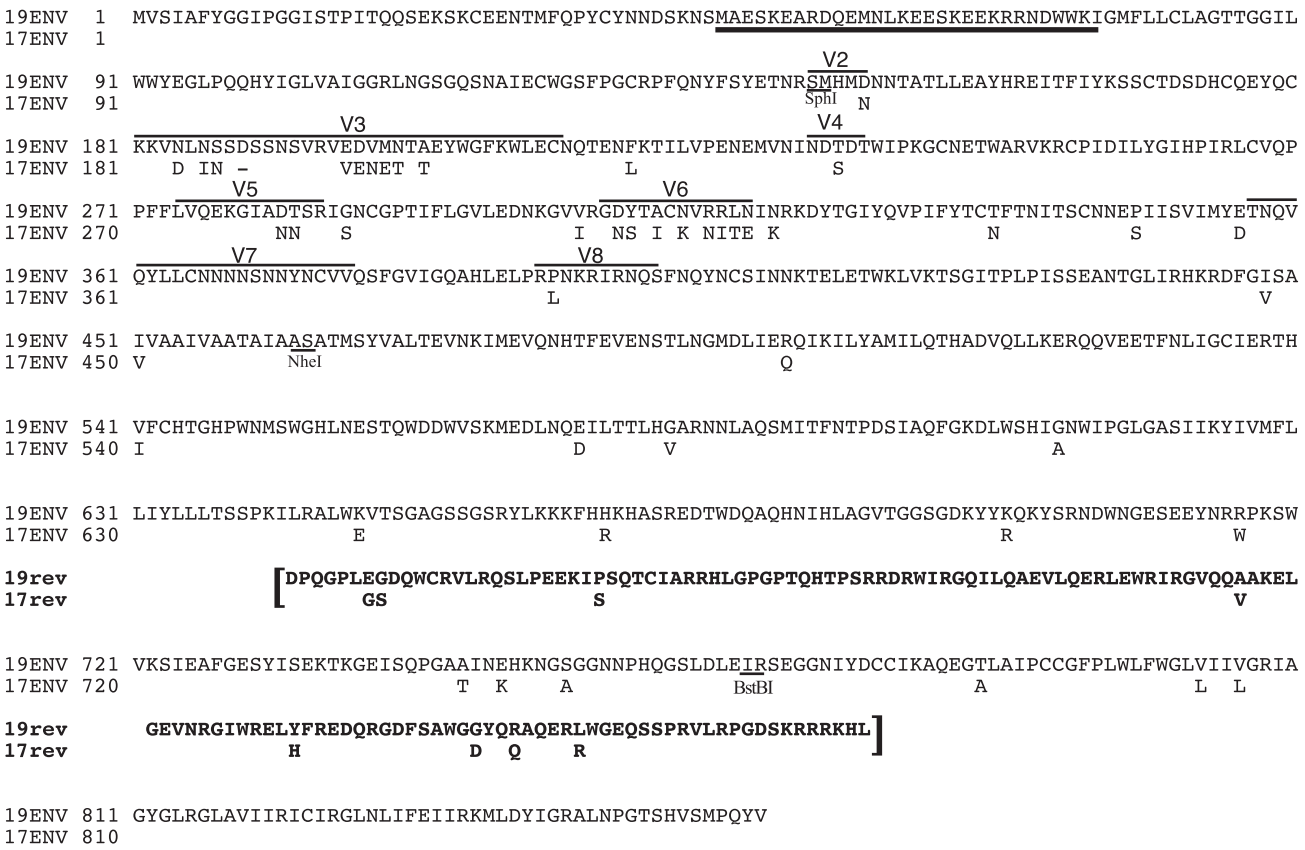

FIG. 3. SU, TM, and Rev amino acid comparisons. The $EIAV_{19}$ sequence is shown above; $EIAV_{17}$ residues that differ are shown below. V2 through V8 indicate previously defined variable regions of EIAV SU (24). The approximate positions of the *Sph*I and *Nhe*I restriction enzymes used to construct $EIAV_{17SU}$ and $EIAV_{17TM}$ are shown. Coding exon 1 of *rev* is in the same reading frame as SU and is shown by the heavy underline. Exon 2 of *rev* is in an alternate reading frame, and the translation is shown in the bracketed region.

than a dose of $EIAV_{17}$ that induced acute clinical disease; however, none of the $EIAV_{19/WYOLTR}$-infected animals developed even mild clinical disease. The combined results from these four infections strongly suggest that both the Wyoming-derived LTRs and the Wyoming-derived *env* gene each contribute to the virulence phenotype of $EIAV_{17}$.

Contribution of SU and TM sequences to virulence phenotype. $EIAV_{17}$ and $EIAV_{19}$ differ by 30 amino acids in SU and 17 amino acids in TM, as shown in Fig. 3. Therefore, we next examined the relative contribution of SU versus that of the TM/*rev* region to the virulence phenotype of $EIAV_{17}$. We constructed p17Nhe/19/wyoLTR and p19Nhe/17, which contain only the SU or only the TM/Rev region from the virulent parent, respectively (Fig. 1). Both constructs derive LTRs from the virulent parent and share common *gag*, *pol*, *tat*, and S2 sequences with both parents. The virus derived from p17Nhe/19/wyoLTR (designated $EIAV_{17SU}$) shares amino acids 147 to 444 of SU with $EIAV_{17}$. Note that the first 438 bp of SU is shared by both the virulent and avirulent parents and that this region encodes the first 146 amino acids of SU as well as the first coding exon of *rev* (Fig. 3). The first 19 amino acids of TM of $EIAV_{17SU}$ are also derived from the virulent parent, while the remainder of TM and the second coding exon of *rev* are from the avirulent parent. $EIAV_{17SU}$ was used to infect Shetland ponies (results shown in Fig. 4E and F). Both ponies experienced acute febrile episodes. Pony E2063 had a single febrile episode at days 10 to 12 p.i. The febrile episode resolved, and the animal maintained a normal body temperature for the next 2 months. Pony E2064 also experienced acute disease but with somewhat different characteristics. A day of elevated body temperature was recorded on day 10 p.i. and again at day 20 p.i. A more notable and extended febrile episode occurred at days 30 to 32 p.i. The body temperature then remained normal through the next 75 days p.i. While disease episodes were observed, it should be noted that they were less severe than those previously observed in ponies receiving $EIAV_{17}$ (Fig. 4A) (37); note also that the infecting dose of $EIAV_{17SU}$, as determined by two different infectivity assays (ID_{50} and initial provirus copy number), was up to 30-fold greater than that of the $EIAV_{17}$ parent (Table 1).

$EIAV_{17TM}$ (from construct p19Nhe/17 [Fig. 1F]) shares amino acids 20 to 417 of TM and shares its overlapping *rev* sequences with virulent $EIAV_{17}$. The clinical profiles of two animals infected with this virus are presented in Fig. 4G and H. These animals seroconverted at 10 and 21 days p.i., similar to the results observed for $EIAV_{17SU}$-infected animals, but body temperatures remained normal for more than 50 days p.i. Results of infectivity assays show that at an inoculum of 2×10^5 cpm of RT activity, the ponies received 6- to 20-fold more infectious virus (as measured on eMDM by ID_{50} and provirus

TABLE 1. Maximum titers and relative infectivity of virus stocks

Virus designation	Maximum RT titer (cpm/ml)	Relative ID_{50} per cpm[a]	Relative provirus copy no. per cpm[b]	Pony inoculum (cpm)	Relative infectious virus dose administered
$EIAV_{17}$	$10^{5.0}$	1	1	5×10^4	0.5
$EIAV_{17}$	$10^{5.0}$	1	1	10^5	1
$EIAV_{19/WYOLTR}$	$10^{4.9}$	0.1	ND	2×10^5	0.2[c]
$EIAV_{17E/19L}$	$10^{5.0}$	1.25	ND	2×10^5	2.5[c]
$EIAV_{17SU}$	$10^{4.6}$	30	16	10^5	16–30[d]
$EIAV_{17TM}$	$10^{4.8}$	10	3	2×10^5	6–20[d]

[a] The ID_{50} per cpm for the $EIAV_{17}$ virus stock was 2.
[b] In a single experiment, the provirus copy numbers per cpm were 580 (±560), 9,000 (±3,600), and 1,660 (±640) for $EIAV_{17}$, $EIAV_{17SU}$, and $EIAV_{17TM}$, respectively.
[c] Based upon ID_{50} determination.
[d] Range is based on ID_{50} and provirus copy number determinations.

copy number) relative to the lethal dose of $EIAV_{17}$. This finding indicates that the TM/*rev* region alone is not sufficient to account for the observed virulence phenotype of $EIAV_{17}$. By all criteria used to determine the titer of our virus stocks, both $EIAV_{17SU}$ and $EIAV_{17TM}$ were administered to ponies at doses equivalent to or higher than the lethal dose of $EIAV_{17}$, yet neither induced acute febrile episodes requiring euthanasia.

DISCUSSION

We generated a series of infectious molecular clones of EIAV in order to map the major virulence determinants of this lentivirus. Our focus was to identify genetic regions that influence the development and/or severity of acute disease. These studies were facilitated by the availability of genetically related clones (pSPeiav19 and p19/wenv17) that produce viruses ($EIAV_{19}$ and $EIAV_{17}$) carrying different virulence phenotypes. The parental viruses and all of the new chimeric viruses replicate in eMDM with cytopathic effects. The RT titers of the new virus constructs were generally comparable to those of the parental clones (Table 1). Pony inocula were based upon RT activity; however, we subsequently determined minimal infectivity titers of the stocks used for the infections. ID_{50}s per cpm were determined by using primary eMDM from a single donor animal. Unfortunately, the life span of eMDM under our standard culture conditions is limited to approximately 3 weeks, an experimental endpoint that might not allow adequate comparison of viruses with similar infectivity but different replication kinetics. To attempt to address this possibility, we performed a single experiment in which provirus copy numbers present at 24 h p.i. were determined by real-time PCR. As shown in Table 1, the relative amount of provirus at 24 h p.i. per cpm of inoculum paralleled the ID_{50}s per cpm for $EIAV_{17}$, $EIAV_{17SU}$, and $EIAV_{17TM}$. With one exception ($EIAV_{19/WYOLTR}$), the ID_{50}s of the tested viruses were equivalent to or greater than that of the parental $EIAV_{17}$ virus. Since none of the chimeric viruses produced disease severity equivalent to that produced by $EIAV_{17}$, it is clear that the ability to replicate in eMDM per se is not a helpful indicator of the potential for the development of severe acute disease by EIAV.

Our in vivo assays consisted of using a small number of experimental animals and performing infections with high doses of virus. Using these parameters, we showed that both the *env* and LTRs derived from the Wyoming field strain were required for the virulence phenotype of $EIAV_{17}$. Replacement of either the Wyoming-derived LTRs or *env* with those of the avirulent strain yielded a virus that caused no acute disease in our experimental system. $EIAV_{17}$ and $EIAV_{17E/19L}$ differ only in their LTR sequence and display dramatically different virulence phenotypes. The two LTRs have multiple sequence differences that alter transcription factor binding motifs (Fig. 2). Detailed descriptions of EIAV LTR sequences similar to those in the present study have been reported previously (28, 29), but to our knowledge, direct in vivo comparisons of these two LTRs have not been described. We have previously demonstrated that the Wyoming LTR neither drives transcription of a reporter gene construct nor supports virus replication in fibroblasts (36). This finding explains the selection of variant LTRs (similar to the $EIAV_{19}$ LTR) during adaptation to growth in fibroblasts. It has also been shown that most field strains of EIAV have LTRs that closely resemble the Wyoming strain consensus and that there is little LTR variation when virus containing a Wyoming-like LTR is examined over time in vivo (26). Finally, Wyoming-like LTRs emerge in horses infected with culture-adapted virus stocks (35). These data strongly suggest that Wyoming-like LTR sequences are favored in vivo. Remaining unclear are the critical interactions between viral LTR and host cell that favor the Wyoming LTR in vivo and allow for disease expression from $EIAV_{17}$ but not $EIAV_{17E/19L}$. A higher expression level in vivo in circulating monocytes or tissue macrophages may lead to an increased virus burden in the infected animal; however, an in vitro difference in replicative ability is not apparent. Alternatively, the $EIAV_{17}$ LTR might facilitate virus replication in cells other than monocytes and macrophages, and this ability may contribute to the virulence phenotype. In this regard, Maury et al. (30) previously demonstrated the replication of EIAV in primary equine endothelial cells. Replication efficiency was strain dependent, with three of four different strains replicating in umbilical cord and renal artery endothelial cells. Interestingly, the Wyoming strain of EIAV was the one tested strain that failed to replicate in these cells (30). It is also possible that subtle but important differences in the regulation of virus expression in vivo are affected by the LTR sequence. Future fine-structure mapping studies of the EIAV LTR will be necessary in order to define the contributions of specific transcription factor binding motifs to the virulence phenotype of $EIAV_{17}$.

The LTR sequence is not the sole virulence determinant of $EIAV_{17}$; the Wyoming-derived *env* gene also contributes to its virulence phenotype. Forty-seven amino acid differences are found in the *env* regions $EIAV_{17}$ and $EIAV_{19}$. Thirty of these are located in SU; 17 differences are found in TM. Rev sequences differ by eight amino acids in the second coding exon. To delineate the differences that exert the highest impact on virulence, we chose to examine *env* in the context of the Wyoming-derived LTR. Using a convenient *Nhe*I restriction enzyme site, we generated $EIAV_{17SU}$, which derives SU, and the first 19 amino acids of TM, from the virulent parent. Due to the method of generating $EIAV_{17}$, $EIAV_{17SU}$ shares most of TM and all of its *rev* sequences with the avirulent parent. A high dose of $EIAV_{17SU}$ caused acute disease in our two test animals. However, the disease was not as severe as animals

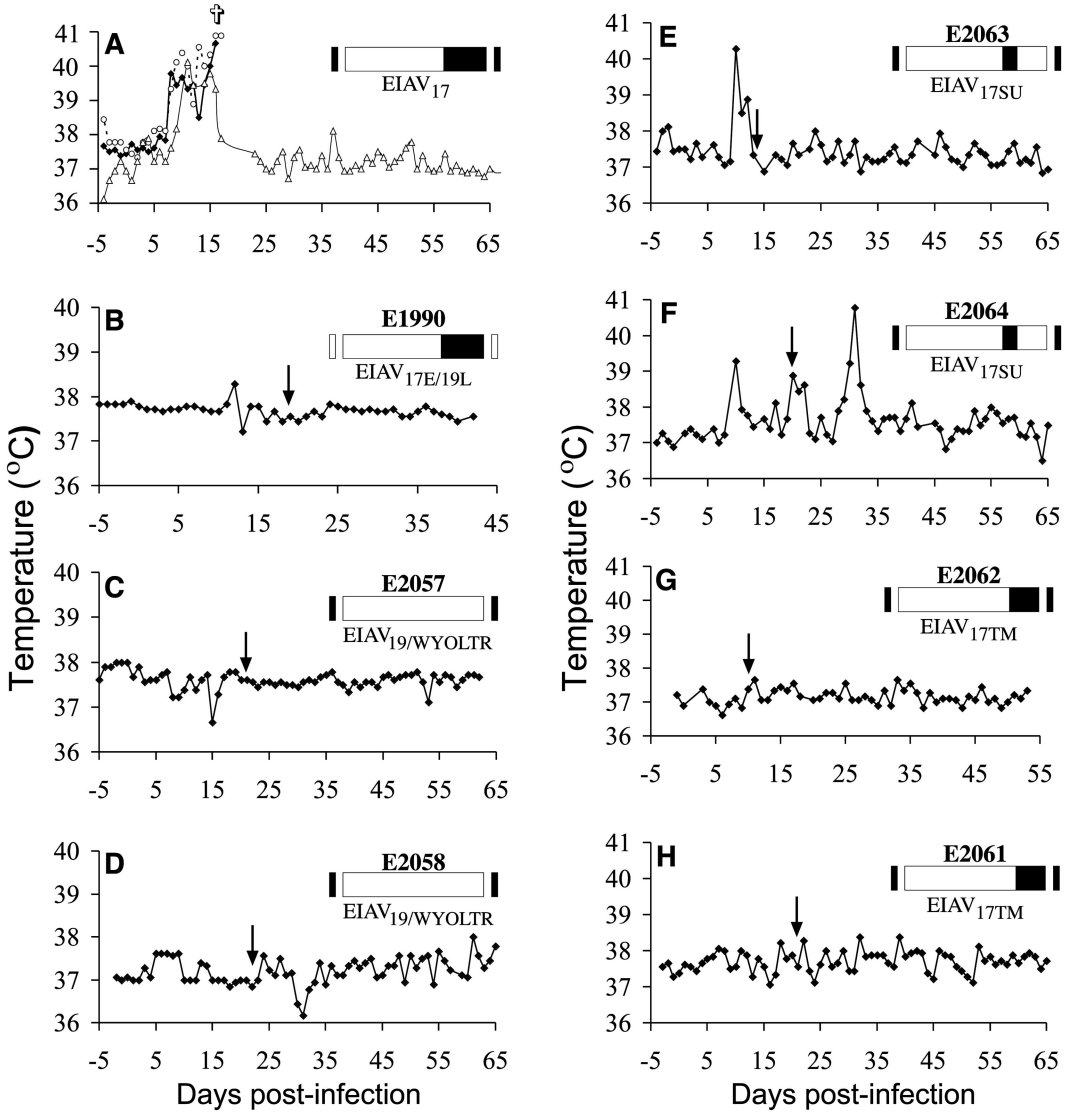

FIG. 4. Clinical profiles. (A) $EIAV_{17}$-infected ponies. The infecting doses for ponies 38059 (○) and 29987 (♦) were 10^5 cpm of RT activity; pony 29990 (△) was infected with 5×10^4 cpm of the same $EIAV_{17}$ virus stock (37). Ponies 29987 and 38059 were euthanized at 17 and 18 days p.i., respectively. (B) E1990 was infected with an eMDM culture supernatant containing 2×10^5 cpm of RT activity of $EIAV_{17E/19L}$. Body temperature remained normal for 45 days p.i. The arrow indicates the day of seroconversion as determined by ELISA. (C and D) Ponies E2057 and E2058 were infected with eMDM culture supernatants containing 2×10^5 cpm of RT activity of $EIAV_{19/WYOLTR}$. Body temperatures remained normal for 65 days p.i. with no other clinical abnormalities noted. Arrows indicate the days of seroconversion as determined by agar gel immunodiffusion assay. (E and F) Ponies E2063 and E2064 were infected with 10^5 cpm of RT activity of $EIAV_{17SU}$. (G and H) Ponies E2062 and E2061 were infected with 2×10^5 cpm of RT activity of $EIAV_{17TM}$. In panels E and F, elevated body temperatures indicate febrile episodes that are typical of EIAV infection. Days of seroconversion (as determined by ELISA) are indicated by arrows.

infected with $EIAV_{17}$ (37), even though the $EIAV_{17SU}$ inocula, as determined by ID_{50}, were severalfold greater than those of $EIAV_{17}$. This finding strongly suggests that the presence of TM/*rev* sequences from the avirulent parent attenuates the overall virulence phenotype.

There are 30 amino acid differences in SU between the avirulent virus, $EIAV_{19}$, and both of the acutely virulent viruses ($EIAV_{17}$ and $EIAV_{17SU}$). The clones contain very divergent V3 sequences. V3 is the principal neutralizing domain (PND) of EIAV (2) and is a region of extensive sequence variation in vivo (23, 24, 35, 45, 46). Both insertions and deletions in the EIAV PND are common, and viruses that totally lack the PND have been recovered previously (24). $EIAV_{17}$ and $EIAV_{19}$ also differ in the previously defined V4 through V6 regions of SU (24). V4 is a short variable region of five amino acids (NDTDT in $EIAV_{19}$ and NDSDT in $EIAV_{17}$). Both viruses have a potential N-linked glycosylation site in V4, and it seems unlikely that the T-to-S substitution would play a major role in the virulence phenotype. The V5 region of EIAV has been defined as a 10-amino-acid sequence (24). The V5 sequence of $EIAV_{17}$, but not that of $EIAV_{19}$, contains a potential N-linked glycosylation site. Examination of V5 from animals monitored during experimental infections reveals that V5

frequently contains an N-linked glycosylation site, though its position within the domain may vary (23, 24, 46).

$EIAV_{17}$ and $EIAV_{19}$ also diverge extensively in the V6 region of SU, where they differ by 8 of 13 amino acids (positions 308 to 320). A functionally equivalent substitution of V to I occurs near V6, at position 305, and two functionally equivalent amino acid substitutions occur within V6 (Y309S and A311I). A striking difference in V6 between $EIAV_{19}$ and $EIAV_{17}$ is the presence in $EIAV_{17}$ of two potential N-linked glycosylation sites that are generated by a D-to-N substitution at position 308 and by R-to-N and L-to-T substitutions at positions 314 and 317. Examination of this region in persistently infected animals shows a number of N-linked glycosylation sites in the V6 region that varies from 0 to 2 (23, 45, 46). The two clones have similar V7 sequences and differ in V8 by one amino acid.

The evolution of the V3 and V4 regions of EIAV SU have been previously linked to the development of neutralization resistance phenotypes during long-term infection (17), and a neutralizing monoclonal antibody has been mapped to V5 (2)—strong indications that these regions evolve in response to immune pressures. The V6 region of EIAV SU has not yet been demonstrated to play a role in virus neutralization, as no neutralizing antibodies to this region have been identified (17); however, only a limited number of such antibodies have been tested. There is presently little information regarding specific functions of various SU domains. For example, cellular receptors and/or coreceptors for EIAV have not been identified, and no direct effects of purified EIAV SU on cell function or disease processes have been reported. While the present study suggests a role for SU in the development of acute disease, additional studies are required so as to clarify its role in disease expression.

Both of the acutely virulent viruses ($EIAV_{17}$ and $EIAV_{17SU}$) also share the first 20 amino acids of TM. This region differs between the virulent and avirulent viruses at two positions (I4V and I7V) that lie in the fusion peptide of EIAV TM (based on similarity to SIV and HIV TM proteins). As these substitutions do not alter the hydrophobic nature of the domain, it would be surprising if they played a major role in the virulence phenotype of $EIAV_{17}$.

$EIAV_{17TM}$ shares amino acids 20 to 417 of TM (and the second coding exon of *rev*) with the virulent $EIAV_{17}$. Virus derived from $EIAV_{17TM}$ failed to cause an acute febrile episode in either of two infected animals, suggesting that TM/Rev is not solely responsible for the virulence phenotype of $EIAV_{17}$. Our results do suggest a role for TM/Rev in virulence, however, as $EIAV_{17SU}$ is less virulent than is the $EIAV_{17}$ parent. Comparison of the TM sequences between the avirulent and acutely virulent viruses reveals 17 amino acid differences. None of the amino acid substitutions in TM change the position or number of N-linked glycosylation sites, and hot spots for variation in TM have not been noted.

The role of *env* sequences in lentivirus virulence has been clearly demonstrated in simian-human immunodeficiency virus (SHIV)-infected rhesus macaques (7, 8, 11, 12, 16, 20, 22, 27, 43). As few as 12 amino acid differences in the *env* sequence are sufficient to change pathogenic potential of SHIV clones (43). Most of the *env* changes that arise with increased virulence are localized to gp120 variable regions; changes in the number and position of N-linked glycosylation sites are common (7, 8, 16, 27, 43). Some measurable differences in virulent SHIVs that have been attributed to *env* sequence changes include fusogenicity, resistance to neutralization, and increased replicative potential (7, 8, 43). During the present study, we determined that infectivity of EIAV for eMDM is not a reliable indicator of virulence. However, we have not yet compared the replication kinetics of our virus stocks. In addition, the development of acute disease generally occurs prior to the development of a detectable immune response; thus, resistance to neutralization is unlikely to play a role in this process. The possibility that other *env*-mediated biologic differences correlate with virulence is currently under investigation.

All of the EIAV clones in this study are identical in the first coding exon of *rev*, which specifies the nuclear export sequence. However there are eight amino acid differences between $EIAV_{19}$ and $EIAV_{17}$ in the second coding exon of *rev* (Fig. 3). Belshan et al. (3, 4) analyzed the *rev* quasispecies present over time in a horse infected with the Wyoming strain of EIAV. $EIAV_{17}$ *rev* is very similar (differing by only one amino acid, E37G) to the predominant *rev* sequence in infectious Wyoming virus stocks (3). This *rev* sequence was present during the acute febrile episode, was not recovered during chronic disease, and then reappeared during a long afebrile period (3). In contrast, the *rev* of $EIAV_{19}$ is similar to *rev* variants that predominated during late-stage inapparent carrier infection (800 days p.i.). Both the virulent and avirulent clones thus contain *rev* sequences that have been found in a natural infection but that may confer a different replication potential or phenotype to the virus.

We are continuing to examine the role of *env* in EIAV virulence by generating additional molecular clones and by monitoring *env* evolution in rapid-passage experiments.

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